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FINAL REPORT

19980058

DEVELOPMENT OF BRASSICA NAPUS GERMPLASM WITH SEED OIL LOW IN SATURATED FATTY ACIDS

Funded by
The Agriculture Development Fund

May 2002

Prepared by: Plant Biotechnology Institute (NRC)

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Development of *Brassica napus* germplasm with seed oil low in saturated fatty acids.

Project 98000058

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Final Report

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A. Abstract

Canola oil compares favorably with other oilseeds in terms of saturate content, however, with the trend toward more *Brassica napus* being grown, the average saturated fatty acid content of the crop has increased. The objective of this project was to develop a microspore mutagenesis system to modify the fatty acid profile in *B. napus*, which would allow canola to retain its competitiveness in relation to other oilseeds. Overall, 124 mutagenesis experiments were conducted in which microspores were isolated and treated with Ethyl methane sulfonate (EMS). From these experiments, 31,984 plants were produced. Over two years, seed from 3740 of these mutagenized lines was planted in field trials. Fatty acid analysis of these lines showed saturate levels ranged from 5.10 to 7.71% in 2000, and 5.25 to 7.71% in 2001. The *B. napus* check variety, AC Excel, had a saturate level of 6.67 – 6.79%. Further field evaluation is required to confirm these results.

B. Technical Report

1. Background

Canola oil has gained widespread acceptance as a superior vegetable oil for human consumption because of its fatty acid composition, particularly its low levels of saturates and elevated levels of monounsaturates. Additional modifications could lead to significant advances in the value and competitiveness of canola. Although canola oil compares very favourably with other oilseeds in terms of saturate content, there is increasing competition for developing varieties with reduced saturates, reduced linolenic acid, and elevated oleic acid in competing oilseeds (especially soybean).

Brassica napus represents about 98% of western Canada's canola acreage [DeClercq, 2001]. With this continuing trend towards more *B. napus* being grown, the average saturated fatty acid content of the crop has increased. This has become a concern because in order to maintain a "low in saturated fat" designation, the total saturates must be lower than 7%. For 2001, overall total saturates was 7.2%. This is an increase from 2000. For the Saskatchewan harvest, saturated fatty acid levels were also 7.2% [DeClercq, 2001].

It is, therefore, imperative that new desirable fatty acid modifications be achieved in canola species to maintain and improve its competitive position in relation to other oilseeds.

2. Project Objectives

The objectives of this project were to:

a. Develop a microspore mutagenesis system and an *in vitro* screening method for detecting lines with altered fatty acid profiles.

b. Screen exotic germplasm for variation in low saturate levels.
c. Facilitate the distribution of this germplasm into the Canadian canola industry in general with emphasis placed on technology and germplasm transfer to Saskatchewan based canola breeding organizations.

3. Results and Discussion

a. Donor Plants

A *Brassica napus* cultivar, Topas, and four breeding lines, TO97-2268, TO97-2288, 3197-2 and 3234-9 from Dr. G. Rakow's *B. napus* breeding program at Agriculture and Agri-Food Canada (AAFC) Research Centre in Saskatoon were grown as donor plants for microspore mutagenesis experiments. Topas 4079 is a highly embryogenic line and was used to develop the mutagenesis system. The four breeding lines were chosen because they possess low saturate levels in comparison to other cultivars. Breeding line TO97-2268 is a double haploid line that has a total saturates level of 6.5%, while line TO97-2288 has low levels of linolenic acid and low saturate levels. Lines 3197-2 and 3234-9 possess total saturate levels of 5.7% and 5.8%, respectively, with 3197-2 produced from F_8 seed from low 18:3 (linolenic acid) parent lines and 3234-9 produced from F_6 seed.

Donor plants were all grown in growth cabinets with a 16 hour photoperiod and 20°/15°C day/night temperature. Just prior to bolting, the temperature was lowered to 10°/5°C. Plants were watered three times per week with 0.35g/l of 15-15-18 (N-P-K) fertilizer.

b. Microspore culture

i. Basic protocol

Microspores were isolated from buds 3-4 mm in size, washed three times in B5-13 [Gamborg, 1968] and cultured in NLN [Lichter, 1982] media supplemented with 13% sucrose (NLN-13). Cultures were then incubated in the dark at 32°C for 72 hours before being moved to a 24°C incubator where they remained for 3 weeks. The embryos were then counted.

ii. Determination of embryogenic frequency

As with many tissue culture systems, there are genotypic differences in microspore embryogenesis response. Although *B. napus* is considered embryogenic in comparison to other *Brassica* species, differences do occur among genotypes. Differences in embryogenesis among plants also occur, especially in segregating populations. Experiments using the *B. napus* microspore protocol [Ferrie, 1995] were first conducted with all four lines to determine the frequency of embryogenesis. Topas 4079 is a highly

embryogenic line, producing thousands of embryos per one hundred buds, and was used as the control.

Response to microspore culture was not consistent between experiments and differences among the four lines were observed in terms of the quality and number of embryos produced (Table 1). Inconsistencies may be due to a heterogeneous population, as these four lines are not true breeding lines.

Table 1 – Embryogenesis of four *B. napus* genotypes

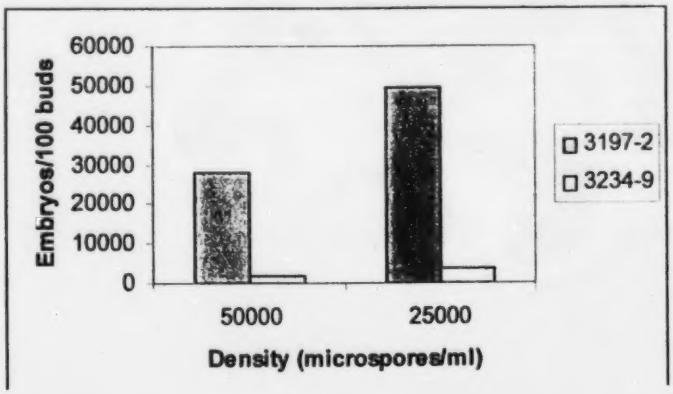
Genotype	Embryogenesis (Embryos/100 buds)
TO97-2268	1083
TO97-2288	2116
3197-2	141304
3234-9	2807

Breeding line 3197-2 was the most embryogenic, producing over 140,000 embryos per 100 buds. However, line 3197-2 still cannot be considered as embryogenic as Topas. Results from previous experiments with Topas have shown that 5-10% of the microspores develop into embryos with projections of up to 50% of microspores undergoing embryogenesis [Huang, 1989; Kott, 1988; Pechan, 1988].

iii. Microspore density

The density at which the microspores are cultured can influence the frequency of embryogenesis. This has been shown in a number of species including *Brassica* [Huang, 1990]. In order to enhance embryogenesis, experiments were conducted to evaluate the effect of different microspore densities on embryogenesis in genotypes 3197-2 and 3234-9. Generally, a density of 50 000 or 100 000 microspores/ml is used but results showed that embryogenesis efficiency could be increased by 100% by using a density of 25 000 microspores/ml when compared with 50 000 microspores/ml (Figure 1).

Figure 1 – The effect of plating density on microspore embryogenesis in *B. napus* genotypes



iv. Media constituents

- **Pluronic F68**

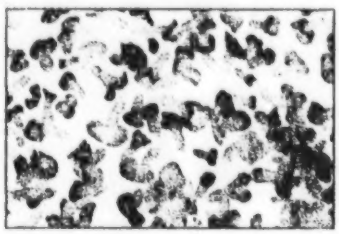
Experiments were conducted to test the effect of pluronic F68 (a co-polymer surfactant) on embryo development and regeneration. Pluronic F68 has been shown to stimulate the growth and division of microspores in wheat cultures [Agricell Report, 2000]. Microspores were cultured in NLN-13 media containing 0.1 mg/ml pluronic F68. Results showed that embryos and plantlets produced from the pluronic F68 treatment were not different from those produced from the NLN control.

- **Polyethylene glycol (PEG)**

Experiments were conducted to determine if polyethylene glycol MW 4000 (PEG) and benzyladenine (BA) could improve embryogenesis of lines TO97-2268 and TO97-2288. PEG is a neutral polymer, which has been used as an alternative to sucrose to create a similar osmotic potential in *B. napus* microspore culture induction medium [Ilic-Grubor, 1998]. BA is a growth regulator, which promotes cell division and shoot development.

Differences in embryo development were observed among all treatments involving genotypes TO97-2268 and TO97-2288. Embryos from media containing PEG + BA were very healthy and normal in morphology when compared to other treatments (Figure 2). Embryos from media containing sucrose grew faster than embryos from PEG-containing medium but showed signs of stress. Embryos derived from PEG-containing medium tend to be smaller than those derived from sucrose-containing medium, but they are more similar to zygotic embryos.

Figure 2 – Microspore derived embryos of *B. napus* grown in PEG and sucrose containing medium



Embryos from PEG medium

Embryos from sucrose containing medium

c. Microspore mutagenesis

Initial microspore mutagenesis experiments used the protocol developed during the Canola Council of Canada/IRAP *B. rapa* microspore mutagenesis consortium project (1995-1998). Freshly isolated microspores from all five lines were treated with Ethyl

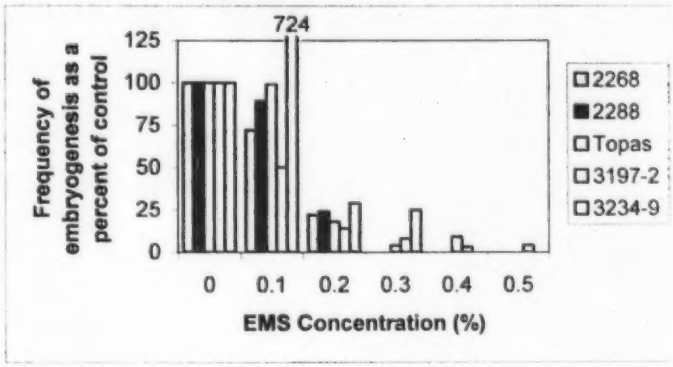
methane sulfonate (EMS), a chemical mutagen, for 1.5 hours. The mutagen was removed by means of a media change and the rest of the experiment was carried out according to the *B. napus* microspore culture protocol.

i. Killing curve

Killing curves for Topas, as well as genotypes TO97-2268, TO97-2288 and 3197-2 were conducted in order to determine the EMS concentration at which 50% and 80% of the microspores were killed (Figure 3).

EMS was applied to the microspores at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5% with all lines, except TO97-2288, which did not receive a 0.5% EMS treatment. The control (0%) received no EMS treatment. Results indicated that EMS was detrimental to embryogenesis in all five genotypes, however, in some experiments, the addition of EMS resulted in an increase in embryogenesis. This was shown by line 3234-9 where an increase in microspore embryogenesis was observed when the microspores were treated with 0.1% EMS (Figure 3). This effect has also been observed in *B. rapa* and *B. juncea* (A. Ferrie, unpublished).

Figure 3 – Effect of EMS on microspore embryogenesis of *B. napus*



EMS affected embryo production in all genotypes and differences were also observed among the lines. EMS killing curve experiments for line 3234-9 were conducted, however, embryogenic frequency in the EMS experiments was very low and while the results have been reported here, they cannot be considered reliable. Using a 0.1% treatment of EMS, approximately one half of the microspores were killed in genotype TO97-2288 and about 20% in genotype TO97-2268. At 0.2% EMS, 99% of microspores in TO97-2288 and 86% of microspores in TO97-2268 were killed. Results from experiments done with genotypes 3197-2 and 3234-9 also indicate that as the concentration of EMS increased, embryogenesis decreased with 0.4 % and 0.5 % EMS treatments being detrimental to genotype 3234-9 (Figure 3). Topas embryos appeared nearly unaffected by a 0.1% EMS treatment producing results similar to that of the

control. A 0.2% EMS treatment, however, gave approximately an 80% reduction in embryogenesis. Since it was not necessary to exceed a 0.2% EMS concentration to produce a 50 - 80% kill, routine experiments were conducted using 0% (control), 0.1% and 0.2% EMS treatments.

A total of 124 microspore mutagenesis experiments were conducted over three years. There were 29 mutagenesis experiments done with genotype TO97-2268 and 19 done with TO97-2288. Genotypes 3197-2 and 3234-9 were used in 29 and 37 experiments, respectively. There were 10 experiments done with Topas.

ii. Colchicine treatment

A method of chromosome doubling is required, as spontaneous doubling rates in *B. napus* microspore cultures are low. Often colchicine treatment is used at the plantlet stage however *in vitro* colchicine applications have been previously used to make the doubling protocol more efficient [Hansen, 1996; Iqbal, 1994; Zaki, 1995].

Colchicine was added to the NLN-13 induction media at a concentration of 10^{-4} M for a period of 72 hours and then removed with a media change. The effect of colchicine and EMS on embryogenesis was evaluated for genotypes TO97-2268 and TO97-2288 (Table 2). Results showed that colchicine was not detrimental to embryogenesis and that embryo yields were higher with colchicine in the induction media.

Table 2 – Effect of EMS +/- colchicine on embryogenesis of genotypes TO97-2268 and TO97-2288

	TO97-2268		TO97-2288	
EMS concentration (%)	+ colchicine	– colchicine	+ colchicine	– colchicine
	Embryos/100 buds			
0	6597	NA	7247	3640
0.1	5331	1970	3812	1127
0.2	940	NA	77	211

The main objective for using colchicine in the induction media is to get an effective and efficient chromosome doubling technique. Results showed that the *in vitro* application of colchicine does result in a high percentage of doubled haploids (Table 3). Doubling rates of up to 86% were attained, however, there were differences between genotypes.

Table 3 – Effect of *in vitro* colchicine treatment on the production of double haploids of *B. napus* breeding lines

	EMS concentration (%)			
Genotype	**0**	**0.1**	**0.2**	**Average**
	% doubled haploid			
TO97-2268	71	72	75	74
TO97-2288	7	86	42	45
3197-2	26	44	50	40
3234-9	54	72	59	62

d. Embryo development and plant regeneration

i. Basic protocol

Approximately three weeks after microspore induction, the embryos are placed on a gyratory shaker at 22°C (16 hour photoperiod) until they turn green (usually about one week). Once the embryos are green, they are plated on 60x15 mm petri plates containing solid B5 media (1% agar, 1% sucrose, with no growth regulators, pH 5.8) and sealed with parafilm. Plates are placed in the tissue culture room (22°C, 16 hour photoperiod) for continued development for approximately another three weeks.

Initial results for genotypes TO97-2268 and TO97-2288 indicated that plant regeneration frequency for both lines was poor. Regeneration of normal plants ranged from 10% to 30%. A number of factors can influence embryo development and regeneration. Experiments were conducted to evaluate several media constituents and physical culture conditions.

ii. Media constituents

- **Abscisic acid (ABA)**

Abscisic acid (ABA) has previously been used to enhance maturation and desiccation tolerance in *Brassica* embryos [Maquoi, 1993; Peng, 1994; Wakui, 1994]. Experiments were conducted using genotypes 3197-2 and 3234-9 to test the effect of ABA on embryo development and regeneration in solid medium. Embryos were treated with various concentrations of ABA for 5 - 7 days after which they were transferred to solid media containing no ABA. After three weeks, the plates were evaluated to determine the amount of normal shoot formation and normal plantlet regeneration. Results indicated that ABA had no beneficial effect on regeneration. Almost all embryos developed into callus with little or no shoot formation.

- **Polyethylene glycol (PEG)**

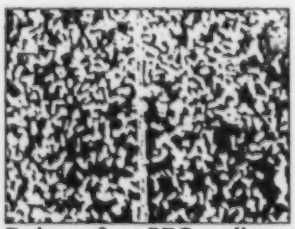
The effect of PEG and BA on embryo development was tested using genotype 3197-2 and 3234-9. As mentioned earlier, PEG is a neutral polymer, which has been used as an alternative to sucrose to create a similar osmotic potential in microspore culture induction medium [Ilic-Grubor, 1998]. BA is a growth regulator, which promotes cell division and shoot development.

Three-week old microspore-derived embryos were either transferred into fresh NLN-13 medium or PEG-based medium. In some cases, additional NLN-13 or PEG was added to the original NLN-13 medium. The control remained in the original NLN-13 medium. Cultures were then placed in the light on a shaker where they remained until green (approximately one week).

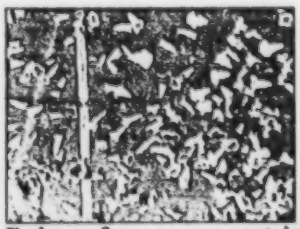
Differences in embryo development were observed for all treatments (Figure 4). Embryos produced from PEG media were very healthy. However, embryos from some PEG treatments were not completely normal in morphology being slightly swollen with relatively short embryonic axes. Media containing sucrose caused embryos to grow faster than those derived from the other media tested. Embryos in sucrose-containing media turned yellow-green and purple which is indicative of stress. This is not uncommon in embryos cultured in sucrose, which is often taken up in larger amounts resulting in abnormalities.

Embryos were transferred onto B5 solid medium for regeneration and it was observed that embryos cultured in PEG + BA medium quickly developed into plantlets which were normal in morphology. Results also showed that the regeneration frequency of those embryos was higher when compared to other treatments.

Figure 4 – Microspore derived embryos produced from PEG and sucrose containing media



Embryos from PEG medium



Embryos from sucrose containing medium

- **Solid media composition**

Comparisons were made between different solid media compositions using genotypes TO97-2268 and TO97-2288. Two regeneration media were compared: A) B5 solid

medium with 1% agar, 1% sucrose and B) B5 solid medium with 0.8% agar and 2% sucrose. Six replicates were done and results showed that medium A produced the best conditions for regeneration with an average frequency of 49.8% while medium B produced an average regeneration frequency of 37.1%. For the remainder of the experiments, all embryos were transferred onto medium A (B5 solid with 1% agar and 1% sucrose) for regeneration.

iii. Physical conditions

Improving regeneration frequency of microspore-derived embryos was the focus of another experiment involving cold treatment and length of time plates were placed on a shaker. Experiments were conducted to evaluate the different combinations of days on a shaker (22°C) and days at 4°C before transferring embryos to solid B5 medium. For genotypes TO97-2268 and TO97-2288, results indicated the regeneration frequency was lower than 20% if the plates were left on the shaker for less than 8 days or more than 42 days with more than 50% regeneration occurring between 15-18 days. The greatest frequency of normal plants regenerated from embryos was 64.6% produced by plates left on the shaker for 16 days.

Storing embryos at 4°C after shaking also improved regeneration frequency. A regeneration frequency of 80.8% was attained by placing plates on the gyratory shaker for 8-9 days and then moving them to 4°C for 23 days.

A similar experiment was done with genotypes 3197-2 and 3234-9 where three week old microspore-derived embryos were placed on a shaker for 5-7 days and then transferred to 4°C for 30-60 days. Genotype 3197-2 was more embryogenic than genotype 3234-9; however, embryos obtained were often small in size and required a supplement of NLN-13 (Figure 5). Embryos from 3197-2 were then placed on the shaker for an additional 5-7 days before refrigeration. This resulted in embryos that were normal in size and morphology. Embryos obtained from experiments involving genotype 3234-9 were normal in appearance and did not require any manipulation before cold treatment was applied (Figure 6).

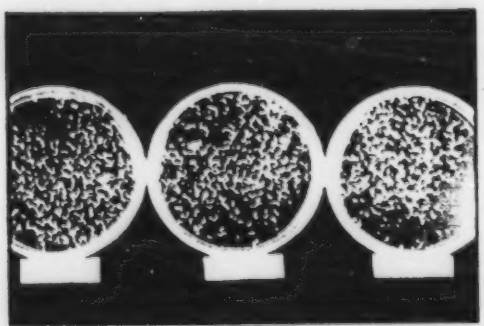


Figure 5 – Embryos from genotype 3197-2

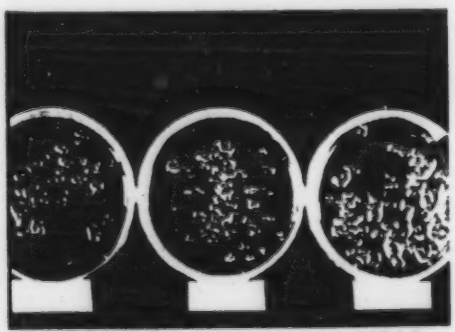


Figure 6 – Embryos from genotype 3234-9

Overall, plant regeneration frequencies were highest for genotype 3197-2 when cold treatment was applied for 40-50 days with frequencies ranging from 45 - 60% compared to 20 day treatments with regeneration frequencies ranging from 25 - 40%. Regeneration frequencies for 15 - 25% were obtained with genotype 3234-9 when cold was applied for 40-50 days with 30 day treatments producing regeneration frequencies ranging from 10 - 20%.

e. Plant development and seed production

i. Basic protocol

Normal plantlets produced from embryo culture were transferred to solid medium containing B5 with 0.8% agar, 2% sucrose at pH 5.8 and placed in a tissue culture room (22°C, 16 hour photoperiod) for further development. Once the plantlets had a well developed root and shoot system (approximately 3 weeks), they were removed from the agar, placed into soil and transferred into the greenhouse (20°C, 16 hour supplementary high pressure sodium lighting) or walk-in growth cabinet (20°C, 16 hour photoperiod).

The majority of plants from all genotypes produced via mutagenized microspore culture were normal and vigorous with good seed production. Overall, genotype TO97-2268 produced 9667 plantlets and genotype TO97-2288 produced 1101 plantlets. Genotypes 3197-2 and 3234-9 produced 12,411 and 6222 plantlets, respectively and Topas produced 2583 plantlets in total. Of the 31,984 plantlets that were regenerated, 12,711 produced seed. The remaining plants did not survive transplanting, were haploid or did not produce seeds. Seeds were harvested from each plant separately once they had reached maturity. The harvested seed weights ranged from 0.001g to 8.96g with the average weight being 1.30g.

ii. Plant production

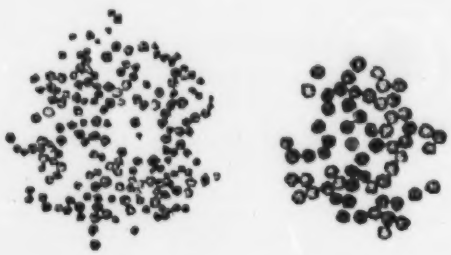
To provide a more efficient method of plant production, large cells (10x10cm) of soil and an ebb-flow system of watering were incorporated into the protocol. Using larger cells versus pots for planting resulted in a reduction in the developmental cycle of the plants by one month. It also saved greenhouse space and transplant time. The ebb-flow system eliminated the need for hand watering and resulted in improved seed set and seed quality in the individual seed lines.

iii. Seed quality

The double haploid plants were improved by means of large scale trimming in the greenhouse. This resulted in less disease and insect damage (Figure 7). The better quality plants produced a higher seed quality and quantity (Figure 8).

Figure 7 – Trimming of plants in the greenhouse improves plant quality

Figure 8 – Seed quality before (A) and after (B) large scale trimming of plants in the greenhouse



A B

iv. Biological controls

In order to keep greenhouse-grown plants pest free and to reduce the amount of pesticides used, biological controls were applied. *Amblyseius cucumeris*, a mite that preys on thrips, was shipped dormant at all life stages and mixed with a food source (bran flakes). The mites were applied by sprinkling the mixture directly onto the plants or by hanging a small paper sack containing the mixture on the stems of the plants (slow release system). The *A. cucumeris* breed inside the sacks and continue to move over the plants for up to 15

weeks. Aphids were controlled by Mummy parasites (*Aphidius colemani*), tiny wasps which parasitize aphids. They were shipped dormant in small bottles and released by opening the bottle and placing it among the plants.

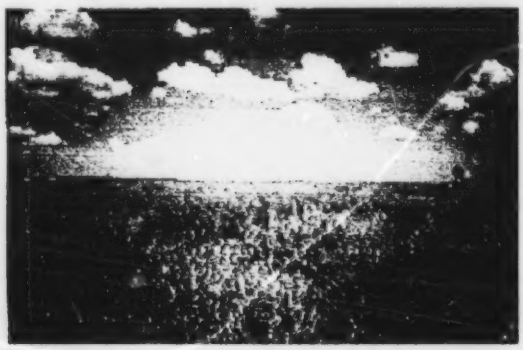
f. Field experiments

Double haploid lines for field evaluation were selected based on the quantity of seed harvested. Individual lines that produced more than 200 seeds were planted in field trials by our collaborating partner (AAFC) in 2000 and 2001 (Table 4, Figure 9). Comparisons were made between the double haploid lines and *B. napus* and *B. rapa* check varieties AC Excel (*B. napus*) and Parkland (*B. rapa*).

Table 4 - The number of double haploid lines grown in the field during 2000 and 2001

Genotype	Number of lines grown in year	
	2000	2001
TO97-2268	1687	1172
TO97-2288	290	6
3234-9	0	241
3197-2	0	344
Total	1977	1763

Figure 9 – Low saturate *B. napus* double haploids being grown in field trials



In 2000, 1977 double haploid lines from genotypes TO97-2268 and TO97-2288 were planted. Two replicates of single rows (100 seeds per row) were seeded on May 13. Genotype TO97-2288 produced poor plants with reduced stand. All lines were harvested individually and fatty acid analysis was conducted.
In 2001, 1763 lines from genotypes 3197-2 and 3234-9 were planted in the field along with genotype TO97-2268 and TO97-2288 (Table 4). Double haploids from genotype

TO97-2268 were vigorous with approximately 90% of the rows being normal in morphology. Genotypes 3197-2 and 3234-9 produced plants with poorer germination and vigor with 30% and 77%, respectively, being normal. Dry, windy conditions resulted in soil drifting which may have been responsible for the uneven germination.

Several differences were observed among the double haploid rows. A number of lines were very late in bolting and appeared to have a winter growth habit but did produce seed. Several other lines flowered very early compared to the checks. Other lines were sterile or had reduced fertility. This was shown by malformation of anthers, petals and stamens.

Seed from 2694 plants will be eligible for 2002 field trials. However, due to staff cutbacks at Agriculture and Agri-Food Canada, these will not be planted.

g. Fatty acid analysis

Fatty acid analysis was conducted on all field grown material. Total saturates were calculated as the sum of C12:0, C14:0, C16:0, C18:0, C22:0 and C24:0. The *B. napus* starting material for the mutation work has about 1% lower total saturated fat content than *B. napus* AC Excel and 0.5% higher total saturated fat content than *B. rapa* Parkland (Table 5). These results are from two years of field testing (1998 nursery and 1999 yield tests at Saskatoon and Scott). All lines were canola quality.

Table 5 - Total saturated fat of the parental material used in microspore mutagenesis experiments and check varieties

Line	Total saturated fat (% fatty acids)
TO97-2268 (DH)	6.4
TO97-2288 (DH)[1]	6.1
TO97-3234 (F_4)	6.3
TO97-3197 (F_4)	6.3
AC Excel	7.4
AC Parkland	5.7

[1] only one year (1997) data, selected for low linolenic acid content.

There were differences observed in the fatty acid levels among the TO97-2268 and TO97-2288 mutagenized lines when compared to the *B. napus* check variety when grown in 2000 (Table 6). Saturate levels of the double haploids ranged from 5.10 to 7.71. There were 832 lines that had a lower saturate level than the parental line TO97-2268 and 64 of those had a lower saturate level than Parkland, the *B. rapa* check variety. Of the 64 lines that had a lower saturate level than Parkland, only one line was derived from the TO97-2288 parent.

Table 6 – Fatty acid analysis of *B. napus* mutagenized double haploid lines, check varieties and parental material grown in 2000.

Fatty acid *	DH (range)	Parkland (*B. rapa*)	AC Excel (*B. napus*)	TO97-2268 parent	TO97-2288 parent
C12:0	0 – 2.17	0.01	0.01	0.01	0.01
C14:0	0 – 0.07	0.04	0.04	0.05	0.04
C16:0	2.79 – 4.43	3.24	3.65	3.49	3.82
C18:0	1.03 – 1.69	1.38	1.82	1.20	1.25
C18:1	47.25 – 61.96	57.85	63.18	56.82	55.95
C18:2	19.06 – 35.63	21.78	17.73	26.01	31.54
C18:3	3.06 – 13.56	12.70	9.29	8.97	3.53
C22:1	0 – 6.19	0.06	0.38	0.05	0.07
Saturates	5.10 – 7.71	5.45	6.67	5.70	6.12

* C12:0 = Lauric, C14:0 = Myristic, C16:0 = Palmitic, C18:0 = Stearic, C18:1 = Oleic, C18:2 = Linoleic, C18:3 = Linolenic, C22:1 = Erucic

The twenty double haploid lines with the lowest saturate levels were all canola quality (i.e. low erucic acid C22:1) (Table 7). Glucosinolates were not evaluated in this experiment. These 20 lines had a similar fatty acid profile as *B. napus* cv. AC Excel, except that there was a slight reduction in oleic acid (C18:1) and a slight increase in linoleic acid (C18:2). The linolenic acid levels ranged from 7.80 to 12.82 (Table 7). The *B. napus* check had a linolenic level of 9.29 (Table 6).

Table 7 - Fatty acid analysis of double haploid lines grown in 2000

DH Line	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C22:1	Saturates
36-107	0	0	3.14	1.07	56.74	23.10	12.29	0.07	5.10
35-73	0	0.04	2.99	1.20	55.85	27.21	8.96	0.07	5.18
15-55	0	0.05	3.31	1.03	51.59	27.89	12.59	0.07	5.20
26-2	0	0.06	3.25	1.06	52.69	27.70	11.59	0.05	5.21
36-619	0.02	0.05	3.14	1.16	56.20	27.27	8.62	0.05	5.26
44-24	0.02	0.05	3.22	1.10	58.52	24.57	9.20	0.05	5.28
15-120	0	0.06	3.25	1.11	58.71	24.90	8.63	0.05	5.30
4-51	0	0.05	3.30	1.12	58.18	25.04	8.75	0.10	5.31
26-15	0	0.05	3.23	1.19	55.99	26.75	9.33	0.07	5.32
38-106	0	0.05	3.44	1.03	55.89	23.35	12.82	0.05	5.32
10-3	0.03	0.06	3.12	1.18	58.50	24.85	8.51	0.06	5.32
24-24	0.03	0.05	3.20	1.17	58.22	25.16	8.77	0.04	5.33
10-32	0.01	0.07	2.79	1.52	60.82	23.10	8.06	0.13	5.33
24-24	0	0.05	3.29	1.17	57.85	25.94	8.28	0.05	5.33
40-24	0	0.05	3.32	1.20	58.49	25.03	8.71	0.05	5.33
10-89	0.06	0.06	3.29	1.07	57.53	25.71	8.94	0.05	5.33
33-51	0	0.05	3.22	1.21	61.07	23.34	7.80	0.04	5.34
35-74	0.03	0.05	3.26	1.16	54.42	25.94	11.44	0.06	5.34
38-229	0.01	0.05	3.31	1.11	56.88	26.13	9.15	0.05	5.35
36-434	0.02	0.05	3.40	1.06	56.37	23.19	12.52	0.05	5.35
AC Excel	0.01	0.04	3.65	1.82	63.18	17.73	9.30	0.38	6.67
Parkland	0.01	0.04	3.24	1.38	57.85	21.78	12.70	0.06	5.45
TO97-2268	0.01	0.05	3.49	1.20	56.82	26.01	8.97	0.05	5.70
TO97-2288	0.01	0.04	3.82	1.25	55.95	31.54	3.53	0.07	6.12

There were also differences in the fatty acid levels among the double haploid lines when grown in 2001. Saturate levels of the lines ranged from 5.25 – 7.71 (Table 8). This is slightly higher than the levels found in 2000. The check varieties, AC Excel and Parkland also showed higher saturate levels when compared to those of the previous year. All lines were canola quality except for one line (69-189) that had 45.71 % erucic acid.

Although there are differences among the double haploids for saturate levels for both 2000 and 2001, the differences are not large enough to suspect a mutation in the biochemical pathway. However, to confirm this, genetic studies would have to be conducted. Further field evaluation would also have to be conducted to confirm the fatty acid profiles.

Table 8 - Fatty acid analysis of *B. napus* mutagenized double haploid lines, check varieties and parental material grown in 2001

Fatty acid	DH (range)	Parkland	AC Excel	TO97-2268	3197-2 parent	3234-9 parent
C12:0	0 – 0.09	0.01	0.02	0.01	0.01	0.02
C14:0	0 – 0.08	0.04	0.04	0.06	0.05	0.05
C16:0	2.52 – 5.08	3.25	3.58	3.44	3.47	3.20
C18:0	0.96 – 2.30	1.43	2.00	1.41	1.88	1.58
C18:1	15.07 – 67.70	57.89	64.91	59.34	61.96	61.71
C18:2	14.16 – 32.29	21.56	17.04	24.67	23.88	26.09
C18:3	2.05 – 12.84	12.71	8.43	7.78	4.97	3.69
C22:1	0 – 45.71	0.13	0.24	0.05	0.06	0.09
Saturates	5.25 – 7.71	5.56	6.79	5.93	6.64	5.93

Table 9 – Fatty acid analysis of double haploid lines grown in 2001

DH Lines	Fatty acid								
	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C22:1	Saturates
61-77	0.00	0.04	2.64	1.51	59.74	28.40	3.26	0.10	5.25
52-58	0.00	0.04	2.94	1.34	60.52	24.24	7.56	0.05	5.28
63-23	0.00	0.04	2.84	1.46	62.98	24.61	4.58	0.04	5.30
46-454	0.00	0.05	3.10	1.31	60.33	24.65	7.34	0.18	5.35
72-5	0.01	0.05	2.57	1.67	62.96	25.05	3.43	0.14	5.36
46-505	0.02	0.04	2.96	1.37	58.73	25.74	7.49	0.04	5.38
82-3	0.01	0.05	2.52	1.73	63.30	24.85	3.37	0.17	5.39
40-79	0.00	0.04	3.05	1.33	63.64	20.36	8.03	0.07	5.41
78-2	0.01	0.06	2.62	1.71	62.21	24.59	4.25	0.43	5.43
69-189	0.00	0.04	2.61	0.96	15.07	14.16	8.37	45.71	5.43
41-160	0.01	0.05	3.12	1.29	58.74	25.59	7.84	0.10	5.44
46-481	0.00	0.06	3.14	1.27	57.30	25.88	8.88	0.16	5.44
63-33	0.00	0.04	3.09	1.36	58.84	28.54	4.53	0.11	5.48
63-51	0.00	0.04	3.03	1.48	61.57	26.15	4.44	0.04	5.49
1-6	0.00	0.04	2.73	1.68	62.43	27.44	2.29	0.04	5.49
43-106	0.01	0.05	3.29	1.22	60.49	23.59	8.10	0.04	5.50
AC Excel	0.02	0.04	3.58	2.00	64.91	17.04	8.43	0.24	6.79
Parkland	0.01	0.04	3.25	1.43	57.89	21.56	12.71	0.13	5.56
TO97-2268	0.01	0.06	3.44	1.41	59.34	24.67	7.78	0.05	5.93
3197-2	0.01	0.05	3.47	1.88	61.96	23.88	4.97	0.06	6.64
3234-9	0.02	0.05	3.20	1.58	61.71	26.09	3.69	0.09	5.93

In vitro screening has been conducted for herbicide and disease resistance as well as for fatty acid analysis with differing degrees of success (Wenzel et al., 1984). Screening for low saturated fatty acids using *in vitro* evaluation, the half-seed method or greenhouse grown material is not reliable. Therefore, it was determined that all double haploids produced from mutagenized microspore cultures were to be increased in the field then the field grown material would be analyzed for its fatty acid profile.

i. Exotic germplasm screening

Exotic germplasm was collected from Agriculture and Agri-Food Canada Research Stations in Saskatoon and Ottawa. Twenty genotypes of forage rape and rutabaga were grown in the greenhouse (Table 8). Seed was harvested and analyzed for fatty acid levels (Table 8).

Table 8 – Fatty acid analysis of germplasm collection

Sample	Species	Source	14:0	16:0	18:0	18:1	18:2	18:3	C22:1
					Fatty acid composition (WT%)				
BCN8311	Rutabaga	ECORC **	0.13	3.64	1.37	21.12	13.92	5.98	40.08
BCN8312	Rutabaga	ECORC **	0.10	4.16	1.11	14.48	15.98	9.39	41.52
BCN8320	Rutabaga	ECORC **	0.09	4.08	0.99	20.07	19.02	10.05	30.69
BCN8327	Rutabaga	ECORC **	0.08	4.17	1.18	13.04	15.24	10.76	42.83
BCN8328	Rutabaga	ECORC **	0.08	4.28	1.46	20.11	14.64	8.93	35.96
BCN8329	Rutabaga	ECORC **	0.08	3.92	1.50	19.47	18.05	8.29	34.22
BCN8331	Rutabaga	ECORC **	0.12	4.09	1.13	11.42	17.63	9.49	44.22
BCN8332	Rutabaga	ECORC **	0.08	3.43	1.56	23.89	12.23	7.14	36.33
SRS2177	Forage rape	AAFC-SK *	0.09	4.81	2.34	66.30	16.48	6.47	
SRS2178	Forage rape	AAFC-SK *	0.09	3.94	1.14	14.65	15.66	6.83	43.04
SRS2179	Forage rape	AAFC-SK *	0.12	5.64	1.93	62.64	20.41	6.27	
SRS2180	Forage rape	AAFC-SK *	0.10	5.70	1.40	57.89	23.02	8.80	
SRS2182	Forage rape	AAFC-SK *	0.10	4.50	2.63	65.91	16.14	7.13	
SRS2183	Forage rape	AAFC-SK*	0.09	5.21	1.65	66.03	17.24	6.73	
SRS2184	Forage rape	AAFC-SK *	0.11	4.82	2.87	63.78	18.93	6.25	
SRS2185	Forage rape	AAFC-SK *	0.14	4.91	2.31	61.98	19.74	7.18	
SRS2186	Forage rape	AAFC-SK *	0.11	5.27	2.03	65.36	16.49	7.20	0.15
SRS2187	Forage rape	AAFC-SK *	0.09	3.93	1.40	18.20	13.48	6.83	41.17
SRS2188	Forage rape	AAFC-SK *	0.13	5.51	2.52	59.99	20.78	7.41	0.16
SRS992	Rutabaga	AAFC-SK *	0.08	3.86	0.96	17.38	0.40	11.86	51.69

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**Eastern Cereal and Oilseed Research Centre – Ottawa, ON

Although a number of forage rape and rutabaga types were evaluated, our focus was on developing low saturate, canola quality *B. napus* double haploids. All the wild *B. napus* germplasm have high levels of erucic acid or high levels of glucosinolates. This was

shown in the sample of lines that were analyzed in this screening study. In order for the germplasm to be beneficial to the breeder and Saskatchewan agriculture, it is essential that the lines be "canola" quality, i.e. zero erucic acid, and zero glucosinolates. According to Kumar and Tsunoda [Kumar, 1980], there is some variation in the fatty acid composition of wild crucifers, however within the tribe *Brassiceae* sub-tribe *Brassicinae*, erucic levels range from 18%-56.7% and saturate (C16:0, C18:0 only) levels range from 4%-14.9%. *Erucastrum abyssinicum* is the species which has the lowest C16:0 and C18:0 levels but it has high a high erucic acid content. This species is not a cultivated crop in Canada. All the zero-erucic acid *B. napus lines* are cultivars, which have been bred for canola quality. This material has already been evaluated for alterations in the fatty acid profiles. Based on discussions with G. Rakow and P. Raney at Agriculture and Agri-Food, we felt that it was more beneficial to start with an agronomic, canola quality *B. napus* breeding line, which has a lower than normal saturate level (derived via conventional breeding) and conduct mutagenesis on this "good" material and then select for those lines with lower levels of saturates.

4. Conclusion

Over the three years, five lines (four breeding lines and one highly embryogenic cultivar) were used for microspore mutagenesis. Embryogenic frequency varied among the lines evaluated. A total of 124 microspore mutagenesis experiments were conducted using 0 (control), 0.1 and 0.2% EMS. Improvements were made to the haploidy protocol (microspore culture, embryo culture, regeneration and plant growth). This resulted in 31,984 plants, of which 12,711 produced seed. From these, 3740 lines were grown in the field and analyzed for fatty acid profiles. Fatty acid analysis with one or two years of field data indicates that there are lines that do have lower levels of saturated fatty acids when compared to the parental lines. These double haploid lines are also beneficial to screen for other characteristics or traits.

A protocol for microspore mutagenesis of *B. napus* was developed and is described as follows:

i. Donor plant conditions

Seeds are planted in 6" pots filled with Redi-earth soil mix. Usually, two seeds per pot are planted and then thinned when the plants are at the 2-3 leaf stage. Slow-release fertilizer (14-14-14 Nutricote) is added to each pot. These pots are placed in a growth cabinet (20°/15°C, 16 hour photoperiod) and watered three times a week with 0.35 g/l of 15-15-18 (N-P-K) fertilizer. When buds are first observed on the plants, the temperature of the growth cabinet is turned down to 10°/5°C.

ii. Microspore culture

a. Selection and sterilization

Approximately 50-75 buds are selected based on size (usually 3-4 mm) and placed in Lipshaw baskets. The buds are surface sterilized in 70% ethanol for one minute and then in 20% bleach (6% Sodium hypochlorite) for 15 minutes on a gyratory shaker. Sterilization is followed by three (5 minute) washes with sterile water.

b. Isolation of microspores

The buds are macerated with a glass rod in 5 ml of half strength B5 medium [Gamborg, 1968] supplemented with 13% sucrose and then filtered through a 44 μm nitex (nylon) screencloth into a sterile 50 ml tube. The glass rod, beaker and filter are all rinsed with 5 ml of B5-13 for a total volume of 20 ml. The crude microspore suspension is centrifuged at 130-150g (1200 rpm) for three minutes, the supernatant is decanted and the pellet is resuspended in 5 ml of B5-13. The centrifugation step is repeated twice more for a total of 3 spins.

c. Mutagenesis (EMS treatment)

After the final centrifugation step, the supernatant is decanted and the pellet is resuspended in 10 ml of NLN-13. The required amount of EMS and microspore suspension are added to three tubes according to the table below:

EMS treatment	**Microspore suspension (ml)**	**Additional NLN-13 (ml)**	**1% EMS (ml)**
0%	2	8	0
0.1%	4	5	1
0.2%	4	4	2
	Total = 10 ml		

The microspores and EMS are incubated at room temperature for 1.5 hours. The tubes are gently mixed periodically to keep the microspores in suspension. After incubation, the microspores are centrifuged (3 minutes at 130-150g) and the supernatant is discarded into 5N NaOH (20g/100ml H_2O). The microspores are then washed three more times with B5-13 as described above.

EMS

To make a 1% solution:

- In laminar flow hood, add 9.9 ml of NLN-13 to centrifuge tube
- In fume hood, add 0.1 ml to the tube
- In laminar flow hood, filter sterilize EMS solution using 10 cc syringe and 0.2 μm filter
- Rinse syringe and tube with 5N NaOH
- Make fresh solution daily

iii. Chromosome doubling

The number of microspores in suspension is determined using a hemacytometer and the required amount of NLN-13 medium containing colchicine (10^{-4}M) is added to the tube to achieve a density of 10^5 microspores/ml. Ten millilitres of microspore suspension is dispensed into each 100x15mm sterile petri plate and the plates are sealed with parafilm.

iv. Culture conditions

The cultures are placed in a 32°C incubator in the dark for 72 hours. The media is then removed from the plates by pipette to a 50 ml centrifuge tube. The tube(s) are centrifuged for three minutes at 130-150g and the supernatant is removed. The same amount of NLN-13 (without colchicine) is added to the tube and the plates are resealed. The cultures are then incubated at 24°C in the dark for the remainder of three weeks. Formation of embryos visible to the naked eye will begin about ten days after induction.

v. Embryo culture

Embryos are counted three weeks after induction. Regeneration can be enhanced by using high concentrations of PEG medium. The determination of the exact concentration to be used would have to be optimized dependent on the genotype being used. The original medium is removed and about 30ml of half strength 25% PEG 4000+Glutamine (0.8g/l)+BA (0.1 mg/l) is added to the plates. The cultures are then placed on a gyratory shaker in the tissue culture room (22°C, 16 hour photoperiod) for about 17 days. Alternatively, the cultures can be placed on a shaker in the light for one week and then moved to 4°C for 2-3 weeks. For regeneration, the embryos are plated onto 60x15mm petri plates containing solid 0-B5 media (1% agar, 1% sucrose, pH 5.8) and placed in the tissue culture room. Embryos that are very small and difficult to pick up individually with forceps can be pipetted onto the solid media.

vi. Plantlet culture

After three weeks, the embryos will have germinated and plantlets will have developed. The normal plantlets are transferred to 150x25 mm petri plates containing solid B5 media (0.8% agar, 2% sucrose, pH 5.8) and placed in the tissue culture room (22°C, 16 hour photoperiod) for further development.

vii. Plant growth conditions and harvest

Once the plantlets have a well developed root and shoot system (approximately three weeks), they are removed from the agar and placed in 10x10 cm cells containing Redi-earth soil mix. The flats of cells are covered with plastic dome lids to maintain humidity and placed either in the walk-in growth cabinet (20°C, 16 hour photoperiod) or the greenhouse (20°C, 16 hour supplementary high pressure sodium lighting). The lids are

slowly removed over a period of five days. The plants flower within the next 1-2 months. The inflorescences are bagged and any haploid plants are recorded and then discarded. Seed set occurs within 3-4 months of transplanting. Seeds are harvested from each plant separately once they have reached maturity. Plant morphology and any unusual characteristics (flat stem, irregular flower color, etc) are recorded. Seed is weighed and recorded.

viii. Future experiments

The double haploid lines developed in this study possessing altered fatty acid profiles will be incorporated into canola breeding programs. These double haploid lines can also be evaluated and screened for other useful characteristics.

C. Personnel

The ADF grant has provided salary for a full time technical officer and a part-time technical officer as outlined below:

Full time technical officers:
April 1999 – October 2001 - Kangfeng Mei
June 2000 – December 2000 – Debbie Doell (to cover K. Mei's maternity leave position)
November 2001 - January 2002 - Marie Mykytyshyn
February 2002 – March 2002 – Heather Macza
Part-time technical officer
September 2001 – March 2002 – Shelley Eftoda

D. Equipment

No major equipment was purchased with project funds. Growth Cabinet space was rented from SOCO at Innovation Place.

E. Project developed materials

The 3740 mutagenized double haploids of *B. napus* have been evaluated for alterations in the fatty acid profiles and can be incorporated into a breeding program. This material can also be evaluated for other traits that are important to a canola breeding program (e.g. disease resistance, insect resistance, agronomic characteristics).

F. Project photos

Photos are included in the final report.

G. Acknowledgement

A paper will be presented at the 13th Crucifer Genetics Workshop in Davis, CA (March 23 – 26, 2002). This paper will discuss the applications of double haploidy and mutagenesis (Abstract is attached). Reference will be made to the work on *B. rapa, B. napus* and *B. juncea*. ADF will be acknowledged in the presentation. In addition, when groups tour the facilities and we discuss the projects underway we acknowledge ADF for their support.

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